New directions in antimalarial target validation

Batista, Fernando A; Gyau, Benjamin; Vilacha, Juliana F; Bosch, Soraya S; Lunev, Sergey; Wrenger, Carsten; Groves, Matthew R

Published in:
Expert Opinion on Drug Discovery

DOI:
10.1080/17460441.2020.1691996

Publication date:
2020

Expert Opinion on Drug Discovery

ISSN: 1746-0441 (Print) 1746-045X (Online) Journal homepage: https://www.tandfonline.com/loi/iedc20

New directions in antimalarial target validation

Fernando A. Batista, Benjamin Gyau, Juliana F. Vilacha, Soraya S. Bosch, Sergey Lunev, Carsten Wrenger & Matthew R. Groves

To cite this article: Fernando A. Batista, Benjamin Gyau, Juliana F. Vilacha, Soraya S. Bosch, Sergey Lunev, Carsten Wrenger & Matthew R. Groves (2020) New directions in antimalarial target validation, Expert Opinion on Drug Discovery, 15:2, 189-202, DOI: 10.1080/17460441.2020.1691996

To link to this article: https://doi.org/10.1080/17460441.2020.1691996

Published online: 20 Jan 2020.

EXPERT OPINION ON DRUG DISCOVERY
2020, VOL. 15, NO. 2, 189–202
https://doi.org/10.1080/17460441.2020.1691996

Taylor & Francis
Taylor & Francis Group

REVIEW

New directions in antimalarial target validation

Fernando A. Batista[a,b], Benjamin Gyau[a], Juliana F. Vilacha[a], Soraya S. Bosch[a,b], Sergey Lunev[a], Carsten Wrenger[b]* and Matthew R. Groves[a]*

[a]Structural Biology Unit, XB20 Drug Design, Department of Pharmacy, University of Groningen, Groningen, The Netherlands; [b]Unit for Drug Discovery, Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

ABSTRACT

Introduction: Malaria is one of the most prevalent human infections worldwide with over 40% of the world's population living in malaria-endemic areas. In the absence of an effective vaccine, emergence of drug-resistant strains requires urgent drug development. Current methods applied to drug target validation, a crucial step in drug discovery, possess limitations in malaria. These constraints require the development of techniques capable of simplifying the validation of *Plasmodial* targets.

Areas covered: The authors review the current state of the art in techniques used to validate drug targets in malaria, including our contribution – the protein interference assay (PIA) – as an additional tool in rapid *in vivo* target validation.

Expert opinion: Each technique in this review has advantages and disadvantages, implying that future validation efforts should not focus on a single approach, but integrate multiple approaches. PIA is a significant addition to the current toolset of antimalarial validation. Validation of aspartate metabolism as a druggable pathway provided proof of concept of how oligomeric interfaces can be exploited to control specific activity *in vivo*. PIA has the potential to be applied not only to other enzymes/pathways of the malaria parasite but could, in principle, be extrapolated to other infectious diseases.

ARTICLE HISTORY
Received 18 July 2019
Accepted 8 November 2019

KEYWORDS
Protein oligomerisation; malaria; drug target validation; phenotypic mapping

1. Introduction

Malaria remains a devastating global parasitic disease. There were an estimated 219 million cases and 435,000 related deaths in 2017 [1]. From the six *Plasmodium* species capable of causing human malaria; *Plasmodium falciparum* and *Plasmodium vivax* pose the greatest global health threat. The former is prevalent in Africa, disproportionately accounting for most malaria cases and deaths globally, whereas the latter is a temperate zone parasite [2]. Currently, the only approved vaccine against malaria is RTS, SA/AS018 [3]. Although this approach has an efficiency of 39% in infants aged 5–17 month after 4 injections/treatments, this rate is still precarious in a global scenario [4]. Moreover, *Plasmodium* is known to develop strategies in evading the immune system when challenged with vaccines over time [5].

Artemisinin-based combination therapies (ACTs) are currently the most effective frontline therapies against *P. falciparum* [1]. These became a potent solution to previously acquired resistance to drugs such as sulfadoxine/pyrimethamine and chloroquine in the mid-1900s [6]. Unfortunately, drug-resistance to artemisinin and its derivatives has emerged in Southeast Asia [7]. This highlights the need for constant basic research into the life cycle and metabolism of *P. falciparum* in order to identify areas of vulnerability in the parasite. Once new targets are identified and validated, they can be used to generate new therapeutics that uniquely target the malaria parasite.

Blood-stage proliferation of the malaria parasite in humans is critically dependent on specific metabolites, and their survivability is believed to be tightly dependent on the availability and metabolism of carbon nutrients. Although many key enzymes in carbon metabolism and related pathways could represent potential drug targets, the target-validation process is far from simple, partly owing to the complex nature of the parasite life cycle [8]. This complexity has led to limited number of tools to properly validate antimalarial targets [9]. Current efforts with genetic approaches, small molecules, and siRNA tools among many others as antimalarial validation probes have shown significant limitations. These constraints call for new innovative, highly specific validation tools [10]. This review is therefore aimed to explore the *pros* and *cons* of the tools that are currently used in the validation of potential antimalarial targets. A spotlight is placed on our recent contribution to the field, the use of the Protein Interference Assay (PIA) as a validation probe. PIA exploits a common feature of most protein complexes – oligomerization –, an shared phenomenon seen in all organisms, present in one- third of all protein structures currently available in the Protein Data Bank (PDB) [11,12] and shared by key enzymes of the metabolic cycle of *P. falciparum* [5].

CONTACT Matthew R. Groves m.r.groves@rug.nl Structural Biology & Biophysics; Carsten Wrenger cwrenger@icb.usp.br Enzyme kinetics & Culturing
*These authors contributed equally

Article highlights

- The malarial parasite is a major threat to populations in the developing world and the emergence of multi-drug resistant strains requires the development of new therapies.
- Developments in *in vitro* target validation in malaria have made significant progress, but there remain pitfalls in each methodology that should be borne in mind during the validation process.
- Potential validation techniques should be as specific for the drug target as possible, with minimal cross-talk with other biological systems.
- Disruption of oligomeric interfaces can be achieved by the introduction of mutant subunits in oligomeric protein complexes.
- Oligomeric PIA represents a valuable addition to the current validation toolbox and opens a new avenue for exploring metabolic pathways in drug target validation.

2. Currently employed antimalarial drug target validation tools

Rational drug discovery mostly begins with novel target identification followed by experimental target validation steps, and assessment of molecular druggability [13]. Target identification is, nowadays, a more easily achievable process than it was two decades ago [14,15]. With the advent of the Human Genome Project and rapid screening technologies, an large number of potential targets associated with most physiological processes have emerged. Drug target validation, on the other hand, remains a very challenging process. From a rational drug design perspective, target validation is a merit-based assessment of a molecular target for therapeutic applications. It addresses whether the target of interest is functionally involved in the cause of a disease and its associated symptoms [14] and thus, its suitability for target-based drug research. This 'validation of essentiality' is hinged on, among other factors, a comprehensive understanding of the disease pathophysiology and the target's mechanism of action, as well as its network of interrelated pathways [16]. However, it is important to remark that, by definition, a therapeutic target is only fully validated if a therapeutic benefit with an acceptable safety window is provided [17]. This review is focused on the validation of essentiality as a starting point for target-based drug discovery rather than the final target validation.

Our group have recently published a manuscript that lists new potential antimalarial targets [18]. The list, which aims to avoid the the propensity of the research communities to focus on a small fraction of the proteome (a phenomenon known as the 'Harlow-Knapp effect'[19]), includes, for instance, components of the mitochondrial electron transport chain (ETC), tricarboxylic acid (TCA) cycle and pyrimidine biosynthetic pathway. All the target candidates mentioned in this publication would benefit from validation studies that could unveil their possible essentiality and consequent potential as therapeutic targets.

Differently from rational target-based drug discovery, antimalarial development often starts from the identification of compounds screened against the blood stages of *P. falciparum*. The screening of large compound libraries, known as phenotypic screens, may revolutionize the drug discovery process. More than 6 million compounds have already been tested against *P. falciparum*, and about 0.5% of these compounds presented an EC_{50} lower than 1μM [20–22]. In fact, rational drug design can be considered more an exception than the rule when it comes to antimalarial drug discovery. According to information available in the literature, all the currently approved antimalarial drugs were not developed in a fully rational manner. These drugs resulted from *in animal* or *in vitro* model studies (e.g. phenotypic screens) instead of studies focusing on the inhibition of known, validated targets. Thus, the mode of action of the identified molecules, as well as the mechanisms of emergence of resistance, are often not fully understood [23,24]. The identification and validation of the enzymes and pathways affected by these molecules could reveal the mechanisms by which resistance arises and/or allow for further medicinal chemistry optimizations [25]. Therefore, the importance of the validation process remains. For example, the identification of genetic mutations that mediate drug resistance with subsequent validation has proved to be a successful route to target malaria [26,27]. Mutational analysis of *P. falciparum* under drug pressure has revealed insights into mechanisms of resistance of most of the commonly used antimalarials, such as chloroquine [28,29], amodiaquine [30], piperaquine [31,32], DHFR inhibitors [33], sulfa drugs [34,35], lumefantrine [36], mefloquine [37], clindamycin [38], atovaquone [39,40] and artemisinin compounds [41,42]. Resistance mechanisms against the 4-aminoquinolines compounds primaquine and tafenaquine, the antibiotic doxycycline and the aryl amino-alcohol compound quinine remain unclear [26]. For *P. vivax*, the scenario remains relatively precarious, with genetic mediators of resistance fully reported only for DHFR inhibitors [43], sulfa drugs [44] and the aryl amino-alcohol compounds lumefantrine and mefloquine [45]. The unveiling of resistance mutations and the changes caused by these mutations, enabled by the development of sequencing and gene editing techniques described below, allows for a deeper understanding into why particular treatments fail and supports the design of more effective antimalarial therapies.

There are several conventional tools for antimalarial target validation. These are dominated by genomic approaches which seek to either knock-in or out of a particular gene that encodes for an essential protein in the life cycle of the parasite [10]. Knockdown via conditional and inducible gene expression tools have also taken center stage in an effort to validate enzyme essentiality in *Plasmodium*, especially in instances where a complete knockout is undesirable [46]. Controlling protein activity at the RNA level with transcript interference and degradation tools has also led to a considerable improvement in target validation. Proteomic approaches have been developed to overcome shortcomings associated with genomic tools unable to deal with the functionally different isoforms of RNA transcripts [14]. The *pros* and *cons* of the currently employed antimalarial validation techniques are discussed below.

2.1. Genomic techniques

Genomic modification techniques, sometimes referred to as 'reverse genetics', employ the principle that the physiological function of a target protein could be validated by

manipulation of its gene expression [14]. Complete gene switch on/off techniques, popularly termed as knock-in or knockout, are commonly used to control protein function at the DNA level. Genetic knockout validation, for example, works on the assumption that the effect of administering a highly specific target inhibitor could be precisely replicated by gene deletion or disruption to halt expression [47]. The use of reverse genetics techniques applied to malaria started with the development of systems for both transient and stable transfection of *Plasmodium* parasites. These had to circumvent the initial difficulties found due to the unstable A/T-rich DNA sequence and thick 4-membrane enveloped nucleus of the parasites [35]. The genomic techniques discussed below have been applied to the functional analysis of many *Plasmodium* genes and gene products involved in erythrocyte invasion, sexual differentiation and cytoadherence of infected erythrocytes (Table 1).

2.1.1. Single and double crossover

A versatile system for the manipulation of the *Plasmodium* genome is the stable transgene expression via homologous, single crossover recombination. By the use of this technique, parasites are transfected with plasmids designed to express a transgene and a selectable marker. Successfully transfected parasites are then isolated by positive drug selection. Within the transfected parasites, crossover events potentially integrate the episomal DNA within the parasite's genomic DNA. The selective drug is then removed from the culture for 3–4 weeks. The absence of the drug forces both integration of the episomal DNA into the parasite's genomic DNA and removal of episomal DNA. Further positive reselection with the drug eliminates the non-integrated forms of the parasite. This cycle is repeated several times until the removal of all episomes and isolation of integrated forms (Figure 1(a)) [57]. Thanks to parasite haploidy [46], a single crossover event leads to knockout of parasites' target genes. But despite the advances brought by this method, it remains a long and inefficient procedure, with a timeframe of approximately 12 weeks required to generate the transgenic parasite [10]. Moreover, the persistence of circular forms of the plasmid is a major impediment for isolation of parasites with chromosomally integrated copies of the plasmid [58]. In order to circumvent these limitations, the double crossover approach was applied to *P. falciparum* [59]. Similar to the single homologous recombination, this method begins with transfection and positive drug selection. The cultures are then submitted to a negative selection by the addition of a second drug. During this step, parasites containing episomes are eliminated due to increased sensitivity to the second drug (Figure 1(b)). The increase of sensitivity occurs due to the presence of genes such as thymidine kinase of Herpes simplex virus [60] and the cytosine deaminase of *Escherichia coli* [61] in the transfected construct. The products of these genes convert a normally harmless metabolite (5-fluorocytosine and ganciclovir respectively) into a toxic one. But although this technique increased the potential for integration events and reduced the time to obtain the selected parasite, it presents a 'bystander' effect [62] which causes parasite death due to the presence of the, theoretically, harmless metabolite in the culture even in the absence of the episomal DNA [10].

2.1.2. Customized ZNFs

The use of customized zinc-finger nucleases (ZFNs) represents a more modern approach for modulating the expression of a target gene when compared to the single and double crossover methods. In this technique, the transfection of parasites with a construct that encodes for pairs of engineered zinc-finger proteins linked to an endonuclease (FokI) leads to endogenous expression of these zinc-finger proteins [64]. After binding to their target sequence on each side of the chromosome, the zinc-finger proteins induce nuclease activity, causing a double-strand break (DSB). This allows for alteration of the target DNA sequence by taking advantage of the homology-directed DNA repair mechanisms at the site of nuclease action [65] (Figure 1(c)). In both *P. falciparum* and *P. vivax*, experiments have demonstrated the generation of ZFN-mediated gene deletions, allelic exchanges, and specific nucleotide alterations in the presence or absence of selectable markers (Table 1). Recently, the use of ZFN gene-editing enabled the characterization of the *Pf*CRT resistant variant in

Table 1. Genomic validation techniques applied to antimalarial target validation.

Technique	Principle	Examples of successfully assessed targets	Pros	Cons
Single Crossover	Induction of crossover events through on and off positive drug selection	cdis1, crep1, Ddis1, Ddis2, Drep1, Drep2 [48]; rap1[49]; emp3 [50];	Generation of knock-in/out/down by single crossover event.	Persistence of circular forms/Time consuming
Double Crossover	Induction of crossover events through negative selection	dhfr[51]; p28/p25[52]; CS[53]; p48/45[54]	Increased potential for integration events and reduced the time to obtain selected parasites compared to single crossover.	Bystander effect
Customized ZFNs	Generation of DSBs through binding of specific nuclease-coupled zinc finger proteins to double-stranded DNA	egfp, vps4, egfp, crt [66], pi4k[67]	Generation of genetic alterations in the presence or absence of selectable markers/ reduced the time to obtain selected parasites compared to conventional tools.	High cost
CRISPR-Cas	Introduces DSBs by a Cas9 endonuclease action guided by a single RNA strand	mdr1[74], dhodh[75]; attB, cg6[55]; glo1, glo2[56]	More accurate, faster and cheaper than conventional and ZNF tools	Requires a co-transfection with two plasmids/Lack of cell line with the Cas9 nuclease gene integrated into the genome

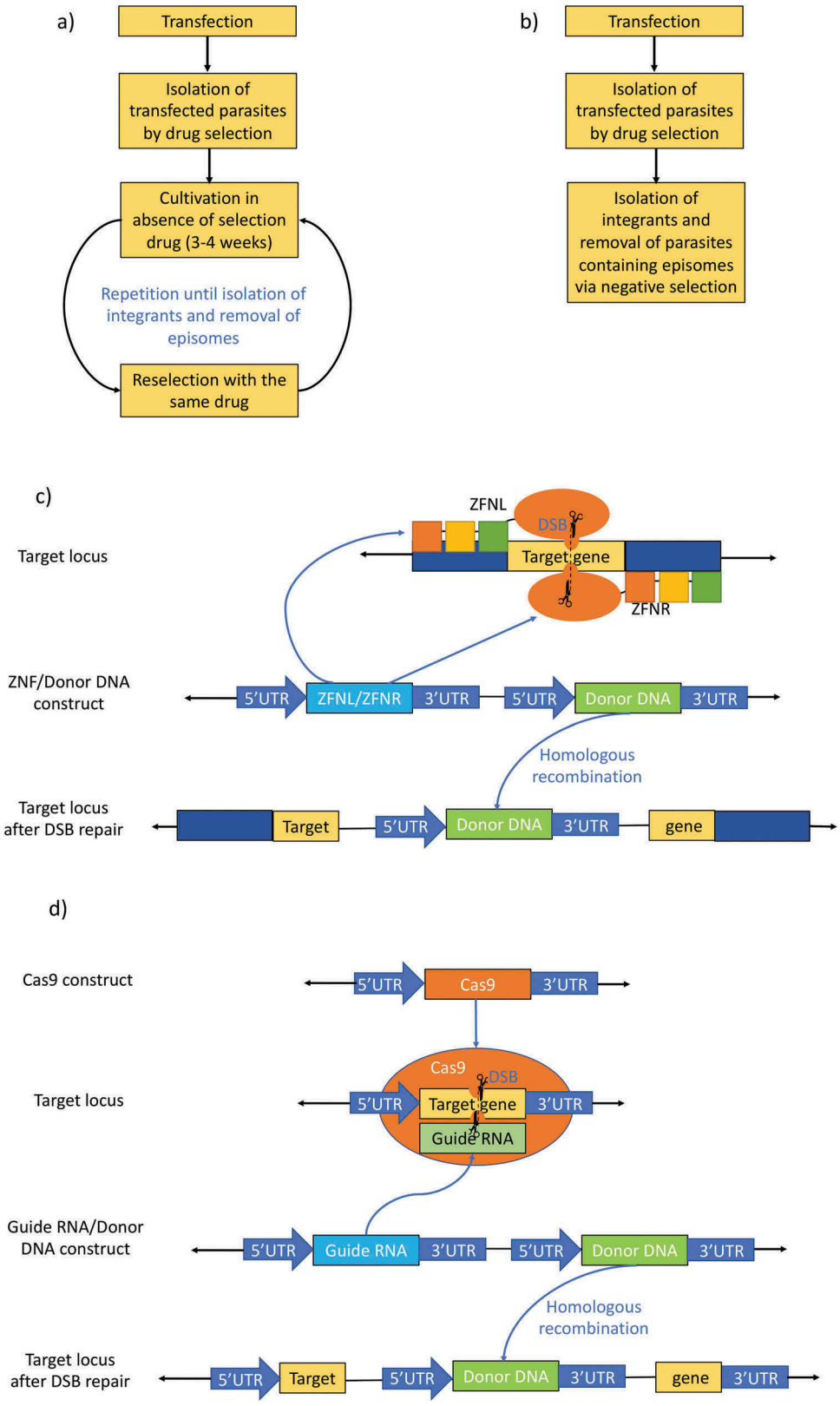

Figure 1. Schematic representation of genomic validation techniques applied to *Plasmodium*. (a) Single crossover [57]. (b) Double crossover [59]. (c) Zinc Finger Nucleases (ZNFs). The parasites are transformed with a construct that encodes for pairs of engineered zinc-finger proteins linked to an endonuclease. After endogenous expression of the ZFNs (showed in the picture as ZFNL and ZFNR) the zinc finger proteins bind to their target sequence on each side of the chromosome and induce nuclease activity, causing a double-strand break (DSB). This allows for alteration of the target DNA sequence by taking advantage of the homology-directed DNA repair mechanisms at the site of nuclease action [64]. (d) Clustered Regularly Interspaced Short Palindromic Repeats-associated proteins (CRISPR-Cas) system. The parasites are co-transfected with two plasmids; one contains the Cas9 nuclease gene, and the other contains the DNA fragment encoding the sgRNA and the donor template DNA. After endogenous expression, Cas9 nuclease forms an RNA-protein complex with the sgRNA. Binging of the sgRNA to the specific genomic locus induces Cas9 to perform a double-strand break (DSB) on the genomic DNA. Homologous recombination between the cleaved genomic *locus* and a donor DNA present in the sgRNA construct leads to the integration of the latest to the genome [63,91].

piperaquine-resistant *P. falciparum* isolates from Cambodia [66]. Another successful example of the use of ZFNs in target deconvolution is the characterization of PI4K mutations that confer resistance to the antimalarial compound class imidazopyrazines [67]. A disadvantage of this approach is the necessity of generation and validation of a new set of sequence-specific ZFNs for each target, which massively increases the cost of the technique [68,69].

2.1.3. CRISPR-Cas

The most recently developed technique in reverse genetics applied to *Plasmodium* is the Clustered Regularly Interspaced Short Palindromic Repeats-associated protein (CRISPR-Cas) system [70]. This method, widely used in multiple organisms, introduces DSBs through Cas9 endonuclease action guided by a single guide RNA (sgRNA) strand (Figure 1(d)). Parasites are co-transfected with two plasmids; one containing the Cas9 nuclease gene, and the other containing the DNA fragment encoding the sgRNA and the donor template DNA. Within the parasites successfully transfected with both plasmids (isolated by positive drug selection), the Cas9 nuclease forms a complex with the sgRNA which contains a sequence complementary to the target gene. This complex binds to the specific genomic locus via the sgRNA and then cleaves the double-stranded DNA via Cas9. Homologous recombination between the cleaved genomic locus and the donor DNA causes integration of the latter to the genome [71–73]. Both gene editing and directional knockout (in non-essential genes) have been achieved using the CRISPR-Cas system (Table 1). The impact of this revolutionary technique can be seen in recent studies that enabled the mechanistic understanding of resistance to antimalarial drugs and drug candidates. Ng et al. have reported the resistance of blood-stage *P. falciparum* parasites against a class of piperazine-containing compounds after successful modification of the gene pfmdr1 [74]. However, this modification promotes increase efficiency of artemisinin-based combination therapy partner drugs [74]. White et al. have used the CRISPR-Cas system to study resistance development against the drug candidate DSM265. Five of the generated point mutations in the binding pocket of the target protein, dihydroorotate dehydrogenase (DHODH), have shown to reduced potency of this compound to inhibit *P. falciparum* growth, suggesting the necessity of a combinational therapy even before the drug hit the market [75].

But while more accurate, faster and cheaper than conventional and ZNF tools described above, the CRISPR-Cas method possesses limitations. As described above, the most traditional approach for the application of CRISPR-Cas in *Plasmodium* parasites requires co-transfection with two plasmids [71–73]. This is necessary due to the absence in *Plasmodium* of the error-prone non-homologous end joining (NHEJ) mechanism, although present in human cells [76]. Instead, the malaria parasites rely on the homologous mechanism [77]. The dependence on whether the two plasmids co-exist in the parasite is a limiting factor for the effectiveness of genetic modification due to very low transfection efficiency in *P. falciparum* [78]. Aiming to overcome the double transfection limitation, a *P. falciparum* cell line expressing the Cas9 nuclease (*Pf*Cas9) was recently established [78]. This cell line makes use of a centromeric plasmid, which segregates with high efficiency, resulting in stable maintenance over multiple nuclear divisions. Although a cell line which possesses the Cas9 nuclease gene integrated into the genome is still to be developed, the use of *Pf*Cas9 cell line has shown improved efficiency.

2.2. Conditional and inducible tools

In malaria, knocking out constitutive genes is often not adequate in the analysis of functional targets with diverse activity at different stages of parasite development [14]. The key to control gene expression temporally and spatially lies in the use of conditional and inducible approaches [79]. The use of the DD/DDD, Tet-Off, riboswitch and Cre/FLP recombinases systems in the generation of conditional knockouts/knockdowns in *Plasmodium* is discussed below (Table 2).

2.2.1. DD/DDD

Conditional knockdown at protein level can be obtained by the introduction of destabilizing domains to the protein of interest. Integration of FK506-binding protein (FKBP)-based destabilization domain (DD) [88] or an Escherichia coli DHFR destabilizing domain (DDD) [65,89] in fusion with the target protein leads to protein ubiquitylation and degradation by *Plasmodium* degradation machinery due to instability of these domains. However, this instability can be reversed by the addition of stabilizing compounds (Shield 1 for DD fusion proteins and trimethoprim for DDD fusion proteins), which allows for control of the expression at protein level. For the use of DDD fusion proteins, parasites must contain a human DHFR expression cassette in their genome, which confers resistance to trimethoprim. Multiple proteins from *P. falciparum* were analyzed by fusion with DD while both *P. falciparum* and *P. yoelii* were subjects of study by DDD fusion approach (Table 2). The use of DD fusion protein has recently validated essentiality of the P. falciparum Merozoite Organizing Protein (*Pf*MOP). Induction of *Pf*MOP degradation resulted in inner membrane complex formation defect, causing maturation arrest with aberrant morphology and parasite death [90]. But although powerful, the use of destabilizing domains is limited by protein location, since proteins that are secreted are not targeted by the protein degradation machinery of the parasite and thus cannot be targeted via this approach [91].

2.2.2. Tet-OFF system

Transcriptional knockdown with the anhydrotetracycline (ATc)-inducible system provides inducible and conditional transgene expression at a defined stage and time [92]. In this approach, the parasites are transfected with a construct containing a gene that encodes for a transcriptional transactivator domain (TRAD). Via homologous recombination, the TRAD is integrated into the genome of the parasite and placed under the transcriptional control of the target gene promoter, while the expression of the target gene will be controlled by an inducible minimal promoter (tetracycline operator – TetO). The binding of expressed TRAD to the inducible promoter (TetO) and consequent induction of the target gene expression is mediated by ATc. Addition of ATc to the culture leads to inactivation of TetO through the inability of the TRAD to bind the operator, halting transcription of the target gene in a controlled manner (Figure 2(b)). Activation

Table 2. Conditional and inducible validation techniques applied to antimalarial target validation.

Technique	Principle	Examples of successfully assessed targets	Pros	Cons
Fusion with destabilization/ degradation domains (DD/ DDD)	Integration of destabilization/degradation domain in fusion with the target protein leads to protein degradation in the absence of the stabilizing compound	MOP[90], calpain [80]; DOC2.1[81]; FPPS/GGPPS[82]	Conditional knockdown at protein level/Applicable to essential proteins/Reversible	Not applicable to secreted proteins
Tet-OFF system	Integration of a TRAD and an inducible promoter into parasite's genome allows for mRNA expression of target genes by the addition of anhydrotetracycline	plasmepsin V[95], EXP2[96], NPC1L1[83]	Reversible/Constructs simple to generate	Not applicable to *P. falciparum*
Riboswitch system	Integration of Glsm ribozyme sequence into a non-coding region of the target gene induces post-transcriptional degradation of mRNA in the presence of GlcN	Acβ, cPKA [100] Hsp70[84]; ClpP, ClpR[85]	Knockdown of essential genes/ Reversible/Constructs simple to generate	Cytotoxicity of the inducer GlcN
Cre/FLP recombinases	Excision of target gene sequence by conditional expression or dimerization of recombinases	msp1[86]; pkg[87]; sub1[105].	Complete ablation of excised gene/Applicable on essential genes	Excision does not occur in 100% of the parasites

or repression of *P. berghei*'s blood stage essential genes have been performed via this approach (Table 2). The disadvantage here is that the tet-OFF system is not fully functional in the generation of conditional knockouts in *P. falciparum*, the most lethal parasitic form, possibly due to lack of efficiency of the TRAD at recruiting transcription factors in this species [10,91]. However, the use of the TetR (tetracycline repressor) domain, which is part of the TRAD, has been successfully reported in an alternative and recently described method that achieves conditional knockdown by combining the (ATc)-inducible system and RNA aptamers [93,94]. Successful examples of target candidates that had their essentiality validated by this method include the protease plasmepsin V [95], the nutrient-permeable channel EXP2 [96], and the Niemann-Pick type C1-related protein [27].

2.2.3. Riboswitch system

A valuable approach for the generation of inducible gene knockdowns is the use of the riboswitch system. This technique makes post-transcriptional regulation possible by the use of the self-cleaving GlmS ribozyme [97,98]. This RNA molecule,

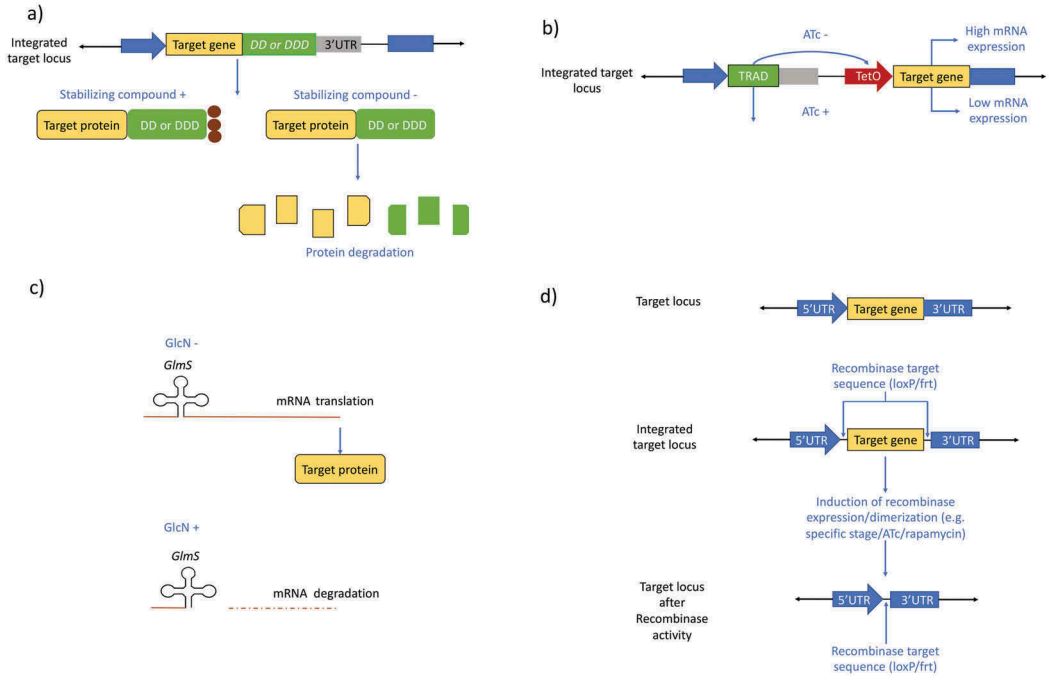

Figure 2. Schematic representation of conditional/inducible validation techniques applied to *Plasmodium*. (a) Post-translational knockdown principle in *Plasmodium*. Integration of FK506-binding protein (FKBP)-based destabilization domain (DD) or an *Escherichia coli* DHFR destabilizing domain (DDD) in fusion with the target protein leads to protein degradation by *Plasmodium* degradation machinery when stabilizing compounds are removed from the culture [65,89]. (b) Tet-OFF system. Integration of episomal DNA upstream the target gene introduces a transcriptional transactivator domain (TRAD) gene sequence and an inducible promoter (TetO). In the absence of Anhydrotetracycline (ATc), the expressed TRAD binds to the inducible promoter, stimulating the mRNA expression of the target gene. Addition of ATc prevents binding of the TRAD to the TetO promoter reducing the expression of the target gene [92]. (c) Riboswitch system. Integration of *Glms* coding sequence to 3'UTR of target gene coding sequence leads to degradation of its transcribed mRNA in the presence of glucosamine-6-phosphate (GlcN) due *Glms* ribozyme activity [97,98]. (d) Conditional knockout. Recombinase target sequences (loxP for Cre recombinase and frt for FLP recombinase) are integrated to the endogenous locus flanking the target gene coding region. The conditional expression of the recombinase encoded by the transfected construct or induction of diCre dimerization results in recombination of the two loxP or frt sites leading to target gene sequence excision [101,102].

in the presence of the inducer glucosamine-6-phosphate (GlcN), can perform cis-cleavage of the mRNA into which they are integrated thereby reducing protein levels [97–99] (Figure 2(c)). The use of this system has been successfully reported in the assessment of multiple *P. falciparum* genes and is currently the method of choice for the generation of inducible knockdowns in malaria (Table 2). For example, a very recent study applied the riboswitch system in the conditional disruption of cyclic AMP signaling pathway components and reported adenylyl cyclase beta (ACβ) and its downstream effector, cAMP-dependent protein kinase (PKA) as critical for RBC invasion [100]. But although highly promising, this technique has as a disadvantage the fact that prolonged exposure of parasites to high doses of GlcN can cause cytotoxic effects [98].

2.2.4. Cre/FLP recombinases

Conditional deletion of gene loci can be achieved by expression of recombinase enzymes such as Cre or site-specific recombinase FLP. These enzymes can trigger the excision of genetic sequences by recombining two target sequences that flank the region to be excised [101,102] (Figure 2(d)). The conditional expression of these enzymes, by systems like the tet-OFF described above, allow for conditional knockout of *Plasmodium* essential genes. Initial studies using Cre recombinase, which recognizes *loxP* sites, have shown low efficiency when combined with a Tet promoter in *P. falciparum*, while FLP, which recognizes *frt* sites, provided better results [65,103]. However, the more recent development of a split diCre protein, which can be induced to dimerize into a functional enzyme by the addition of the ligand rapamycin, has provided control of the expression levels in a more precise manner [104]. A recent example of successful application of this technique includes the study of genes involved in a protease cascade proved to regulate the release of *P. falciparum* parasites from host red blood cells [105]. The conditional knockout of the serine protease SUB1 by diCre caused the parasites fail to rupture the parasitophorous vacuole and preventing the continuation of *P. falciparum* replication cycle [105]. This approach possesses the advantage of providing conditional knockout of *Plasmodium* blood-stage essential genes, with complete ablation of gene function if the gene is excised, but since the excision does not occur in 100% of parasites, the difficulty in distinguishing parasites which gene excision has occurred represents its main downside [91].

2.3. Proteomic approaches

As many gene products have slightly different functional isoforms with further post-translational variations, validating targets at genetic level can be problematic. Arguably, the best approach would be, instead, to perturb the function of the protein of interest [14]. Proteomic validation tools are therefore the ideal in most instances, more so because the majority of developed drugs target specific proteins and not their gene precursors. Although these tools have the advantage of being applicable to parasite essential proteins, structural information of these targets is often required [8]. Here, we discuss the use of aptamers, small molecule validation tools, and our recent contribution – the use of protein interference assay as a new and highly specific tool in the validation of antimalarial target candidates (Table 3).

2.3.1. Aptamers

Aptamers are single-stranded RNA (ssRNA) or single-stranded DNA (ssDNA) oligonucleotides whose unique three-dimensional structure enables them to interact with a specific target [65]. The use of aptamers, also known as Systematic Evolution of Ligands by Exponential Enrichment (SELEX), consists of incubation of a random nucleic acid library with the target culture/lysate followed by isolation of target-bound aptamers and amplification of these aptamers using conserved primer sequences for further rounds of selection [107,108] (Figure 3(a)). The use of negative selection, in which aptamers that do not recognize an alternative target are kept while those that do are discarded, improves the specificity of the selected aptamers and enables the design of a personalized strategy [109]. Although most of the studies that have made use of aptamers in malaria were focused on diagnosis, this technique has potential both in antimalarial target identification/validation and in use as therapeutic molecules [110]. As mentioned above, the recently developed TetR–DOZI–aptamer system combines RNA

Table 3. Proteomic approaches applied to antimalarial target validation.

Technique	Principle	Examples of successfully assessed targets	Pros	Cons
Aptamers	DNA and/or RNA aptamers bind to their target protein inhibiting its biological activity	EMP1[107]; plasmepsin V[95], EXP2[96], NPC1L1[101]	Affinity comparable to monoclonal antibodies/ability to distinguish between protein isoforms and different conformational forms of the same protein	Susceptibility to degradation by nucleases
Small-molecule inhibition	Small molecule-targeting-probes occupy the active site of target demonstrating functional pharmacology and leading to a desired phenotypic effect	cytochrome bc1, DHFR[111]; DHODH[106]	Broad use/Applicable to essential proteins	Often nonspecific for their target of interest/might be limited by rapid metabolism, poor cell membrane passage, and subcellular location
Protein interference assay	Expression of inactive mutant forms of target protein form oligomers with WT protein *in vivo*, reducing their activity and allowing for phenotypic effect analysis	MDH/AspAT[122,124]; ATC (manuscript in preparation)	Applicable on essential proteins/ high specificity	Unable to provide complete knockout effect/Structural information of the target is needed

aptamers and conditional knockout/down elements to enable conditional control of target candidates translation in an efficient manner [93,94].

While aptamers recognize their targets with binding specificities and affinities comparable to those of monoclonal antibodies, the use of the first method possesses multiple advantages over the second one, including lower susceptibility to contamination and no limitation to highly immunogenic targets. Moreover, the small size of aptamers enables them to reach smaller compartments and cells, for which antibody penetration is limited. In terms of efficiency, aptamers require less production time (one to three months) than antibodies (four to six months). As a major disadvantage, aptamers are susceptible to degradation by nucleases. To minimize this problem, chemical modifications or molecules such as polyethylene glycol (PEG) and biotin can be added to their 3′ and 5′ ends to prevent degradation [109].

2.3.2. Small molecule inhibitor probes

Validating targets using pharmacological agents often works in conjunction with computational approaches to leverage the desired high selectivity of chemical probes [15]. Small molecule-targeting-probes must be able to bind the active site, demonstrate functional pharmacology and ultimately lead to a desired phenotypic effect [14]. Many essential proteins of *P. falciparum*, such as cytochrome bc1 and dihydrofolate reductase (DHFR), have been chemically validated. Inhibitors blocking the activity of the latter are of particular interest, as they exploit the folate synthesis pathway, an essential metabolic pathway for the parasite DNA replication process [111]. Dihydroorotate dehydrogenase (DHODH), an essential mitochondrial target enzyme of the *de novo* pyrimidine biosynthesis pathway was recently validated with small-molecule probes [112]. Specific DHODH inhibitors, such as the compound DSM265, demonstrated IC_{50} values of 40–80nM against *P. falciparum* and extremely high specificity for the parasite DHODH over human DHODH [113,114].

Target inhibition by the use of small molecules has also an important role in target deconvolution. As previously mentioned, the identification of resistance mutations selected by the presence of known inhibitors with non-identified targets has the potential to reveal mechanisms of action that can then be targeted by novel antimalarial drugs [26,75,115,116].

But despite the widespread use of chemical probes, their application is still open to confirmational bias as small molecules are often nonspecific for their target of interest [117,118]. In addition, rapid metabolism, poor cell membrane passage and subcellular location after successful entry are still potential disadvantages of chemical probes [15,118].

2.3.3. Oligomerisation interference-based validation

Protein oligomerisation is the self-assembly of more than one copy of a single or different protein(s) into one functional complex [119,120]. Currently, about 60% of all protein structures available in the Protein Data Bank (PDB) are oligomers; dimers been the majority of this class [11,12]. Indeed, oligomerisation is a common feature shared by proteins of all biological systems, and interestingly present in key enzymes of the plasmodial pyrimidine biosynthesis (e.g. orotate phosphoribosyltransferase, OPRT; orotidine 5′-phosphate decarboxylase, ODCase), aspartate metabolism (aspartate aminotransferase, AspAT and aspartate transcarbamoylase, ATC), vitamin B6 biosynthesis (pseudo enzyme, Pdx1.2) and malate metabolism (malate dehydrogenase, MDH) among other carbon metabolism pathways [9,121]. The oligomeric Protein Interference Assay (PIA), our recently proposed validation technique, takes advantage of oligomerization by disrupting oligomeric interfaces using mutant subunits of the target candidates and causing a significant reduction in specific enzyme activity [10,122] (Figure 3(b,c)). The biomechanics of the assembly of oligomer subunits and evolution of oligomeric interfaces make PIA remarkably interesting. Protein oligomerisation has been observed to influence the correct active site or cofactor binding pocket conformation for many proteins, consequently playing an important role in their activity [120,123]. In some cases, residues from different subunits contribute to form the active-site [124,125]. Therefore, interference with a nonfunctional subunit might have a significant effect on the biological activity of these enzymes, as previously demonstrated [124,125].

A recent study was carried out targeting two key-enzymes of the aspartate metabolism pathway, aspartate aminotransferase (*Pf*AspAT, Figure 4) and malate dehydrogenase (*Pf*MDH, Figure 5) [122]. Multiple factors led to *Pf*AspAT and *Pf*MDH to be considered good candidates for the application of PIA based-inhibition for the first time: both enzymes present a low degree of conservation for the residues that form the oligomeric interfaces, unlike the residues that compose the active-site [122,124] and both possess their active sites formed across the interface between 2 monomers, and thus are dependent on their oligomeric state to be fully active [124,126]. Subsequently, *in vitro* experiments have demonstrated that the introduction of mutations on the oligomeric surfaces can impair the function of these enzymes and the mutant forms of both enzymes can form oligomers with the wild-type forms, also impairing their function [122]. In short, endogenous expression of mutant forms of both these enzymes in transfected parasites demonstrated that, although the introduction of AspAT and MDH mutants alone did not result in significant effect on parasite proliferation in blood-stage cultures, the transfection of parasites with both plasmids resulted in a significant reduction in parasite proliferation in aspartate-limited media (used to mimic physiological conditions) [122]. Aspartate is known to be the least common of all the amino acids available within the human serum, with measurements suggesting a concentration of < 20 µM [127]. Although aspartate is available in hemoglobin, which is used as a source of amino acids (except isoleucine) during the blood stage [128], the PIA experiments on AspAT and MDH enzymes suggest insufficiency of this source to support the rapid proliferation of the parasite. Therefore, a functional, aspartate biosynthesis is likely to be a key element for the maintenance of *P. falciparum* in human red blood cells. These data not only validate the aspartate metabolism as an essential pathway in *P. falciparum* but also fully support the hypothesis that oligomeric surfaces offer a highly promising opportunity to specifically influence protein behavior in *Plasmodium in vitro* cultures.

Oligomerisation has the advantage of being highly selective. The evolutionally diverse and large surface area of

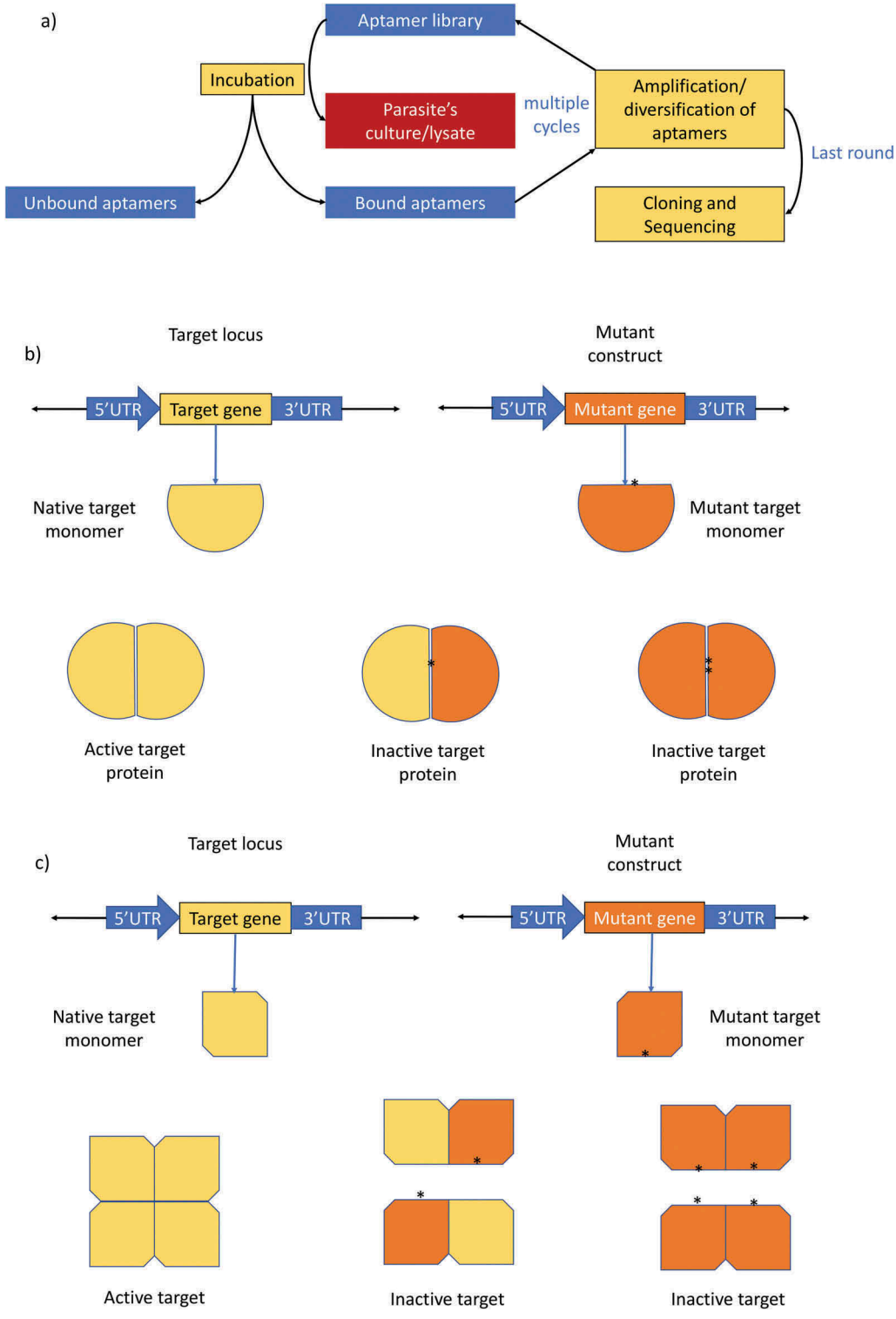

Figure 3. Schematic representation of protein-based validation techniques applied to *Plasmodium*. (a) SELEX methodology. After incubation of the aptamer library with culture or lysate of parasites, unbound nucleic acids are separated from bound ones. Nucleic acid-protein complexes are then dissociated and the nucleic acid pool is amplified and enriched. The new generated nucleic acid pool serves as a starting library for a new SELEX cycle composed of identical steps as the first round. The number of SELEX repetition depends on the library type used and on specific enrichment achieved per a selection cycle. After the last round of aptamer selection, the PCR products are cloned and sequenced [107,108]. (b) and (c) Overview of two independent strategies for the use of Protein Interference Assay (PIA) in *Plasmodium*. In the first, the transfection of parasites with a construct encoding for a copy of the target gene containing one or more inactivating mutations on the oligomeric interface leads to endogenous expression of this mutant. The formation of oligomeric complexes between the native active protein and the mutant protein results in inactive heterocomplexes, leading to a knockdown effect at protein level. In the second, clashing mutation(s) are introduced to one oligomeric interface while other(s) oligomeric interface(s) is preserved. The endogenous expression of the mutant allows for heterocomplex formation with the native protein, preventing the formation of the full oligomeric complex. The presence of the cashing mutation in at least one monomer leads to inactivation of proteins which function depend on the oligomeric state, thus, resulting in a knockdown effect [10,122].

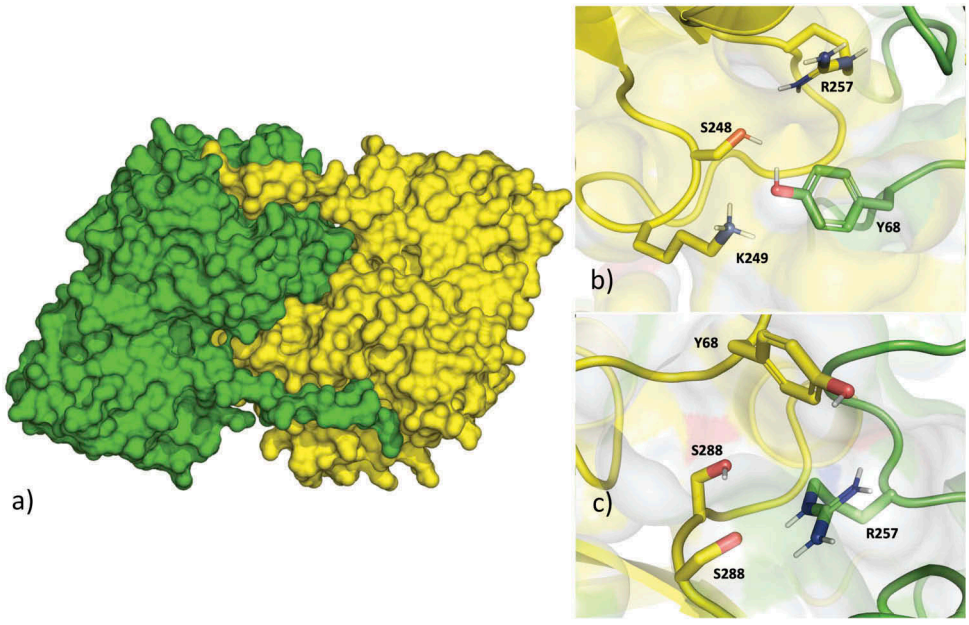

Figure 4. (a) The dimeric structure of plasmodial aspartate aminotransferase (*Pf*AspAT). (b) and (c) The oligomeric interface of the WT-*Pf*AspAT dimer. Residues Tyr68 and Arg257 from both subunits are shown in sticks[122].

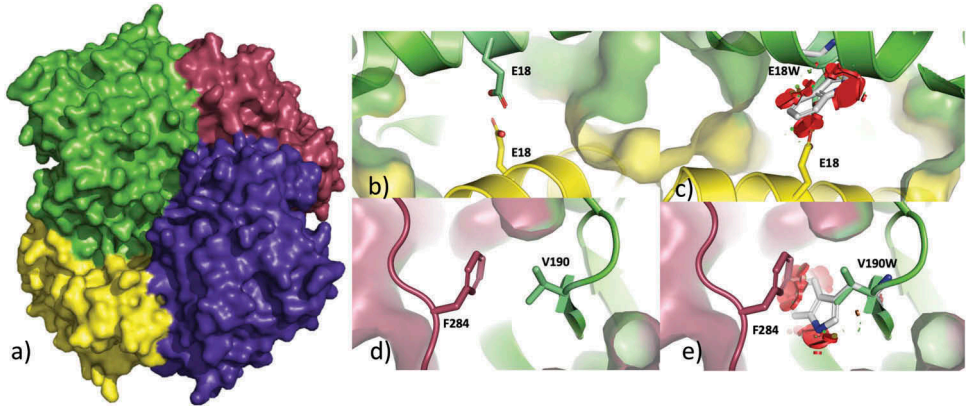

Figure 5. Mutations designed for the protein interference assay (PIA) approach targeting plasmodial malate dehydrogenase (*Pf*MDH). (a) The tetrameric structure of *Pf*MDH, the different subunits are labeled A-D. (b) The A-B interface of the wild-type (WT)-*Pf*MDH tetramer. Residues Glu118 from both subunits are shown in sticks. (c) The steric clash generated by the introduction of a Tryptophan in position 118. The mutant monomer is shown in green and WT monomer is shown in yellow. (d) A-C interface of WT-*Pf*MDH tetramer. Residues Val190 from both subunits are shown in sticks. (e) The steric clash generated by the introduction of a tryptophan in position 190. The mutant monomer is shown in green and WT monomer is shown in magenta [122,124].

oligomeric interfaces ensures unusual selectivity of subunits for each other, minimizing cross-reactivity [123]. Oligomeric surfaces of homologous proteins are, in many cases, significantly less conserved compared to their substrates' active sites and/or cofactor binding pockets [119,123]. In the validation of essentiality of target candidates, this high specificity and selectivity offer an important advantage, for instance, over the use of small-molecule-based validation proteomic tools. The most noticeable disadvantage of this approach is the inability to provide a complete knockout effect since the heterocomplex formation is unlikely to effect all wild-type subunits. The level of inhibition also seems to vary from protein to protein. As an example, specific activity measurements in whole-cell lysates demonstrated an approximately 2-fold inhibition for AspAT-mutant culture compared to control culture, while the MDH mutant showed a slight decrease that did not reach statistical significance when compared to control culture [122]. Furthermore, the design of a PIA experiment requires structural information in order to identify key residues that can induce the desired phenotypic effect.

3. Conclusion

The antimalarial validation toolbox is currently filled with several genetic and proteomic-based tools that can report on a protein's biological activity. However, knockout and knockdown systems are often highly complex or difficult to perform, time-consuming, too costly or are simply not effective for essential genes of the parasite. Moreover, small molecule inhibitor-based tools are often nonspecific and do not always yield the

desired efficacy partly owing to the complex nature of the parasite's life cycle [8]. These and many other challenges with the current toolset highlight the need for novel specific validation alternatives. The validation of *P. falciparum* aspartate metabolism essentiality through the recently developed PIA methodology reinforces the need to revisit the validation processes of targets where conventional validations tools failed.

4. Expert opinion

Possessing a good repertoire of genetic manipulative tools in assessing the role of a gene's product is a must in drug discovery. In this review, the reader was introduced to the currently used validation methods in malaria. As discussed above, despite the particular advantages of genetic techniques, the overall difficulty of applying these methods to essential genes represent a significant drawback for their use as validation methods. In this scenario, conditional and inducible validation tools are of great value. These methods indeed represent a better alternative for validation of essential genes/proteins, but neither of them is applicable to all classes of proteins, all species of *Plasmodium* or possess full efficacy in disturbing/degrading the target. With proteomic approaches, a significant advantage is the ability to assess targets with different functional isoforms with further post-translationally variations, but poor specificity for their target of interest, rapid metabolism, poor cell membrane passage/pentration and subcellular localization after successful entry are still significant barriers. In this scenario, we introduce the concept of the oligomeric Protein Interference Assay (PIA), a highly specific method to control biological activity by disrupting oligomeric interfaces using mutant subunits of the target protein. Via this method, it is possible to achieve a significant reduction in specific enzyme activity in *in vitro* cultures, as demonstrated for the enzymes *Pf*AspAT and *Pf*MDH. Moreover, the previously reported phenotypic effect of the introduction of these mutant subunits within the parasite supported the validation of the aspartate metabolism from *P. falciparum* as an essential pathway without recourse to complex genetic approaches.

It is important to stress that the PIA approach does not represent a replacement for other validation techniques applied to malaria. As other methods, PIA possesses limitations, the most noticeable being the inability to provide a complete knockout effect. Instead of being used as a single definitive method, PIA can be combined with other validation methods to provide a more complete understanding of the assessed target. PIA also represents a powerful method to validate the essentiality of metabolic pathways instead of single targets. When different components are assessed in parallel, as demonstrated for AspAT/MDH, the negative effect in parasites' growth is much more pronounced.

An important addition to the setup of PIA experiments could be represented by the use of bioinformatic tools. Molecular modeling could potentially suppress the need for structural information for some targets. Furthermore, these tools could potentially be used in the development of a pipeline able to identify residues that, if mutated, would cause the desired effect.

Although our report on the PIA approach is, so far, limited to two enzymes of *P. falciparum* [122], the successful assessment of these targets suggests a broader potential for this technique. In principle, the two main requirements for the application of PIA to a specific target are structure information and oligomerization. The constant addition of crystallographic structures to the protein data bank, added to the fact that more than 60% of the structures currently deposited there are reported in an non-monomeric oligomeric state support the hypothesis that the PIA approach can be applied not only to more metabolic pathway within *P. falciparum* but also extrapolated to other infectious diseases.

Funding

The authors acknowledge support from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP grants 2013/17577-9 to SS Bosch, 2017/03966-4, and 2015/26722-8 to C Wrenger). F Batista gratefully acknowledges funding through a Science without Borders Fellowship. Further, the authors would like to acknowledge the CAPES/Nuffic MALAR-ASP (053/14) network.

Declaration of interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

Declaration of interest

No potential conflict of interest was reported by the authors.

References

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. WHO. World malaria report 2018. 210 (2018).
2. Greenwood BM, Fidock DA, Kyle DE, et al. Malaria: progress, perils, and prospects for eradication. J Clin Invest. 2008;118:1266–1276.
3. First malaria vaccine receives positive scientific opinion from EMA | European Medicines Agency. [cited 2019 Oct 28]. Available from: https://www.ema.europa.eu/en/news/first-malaria-vaccine-receives-positive-scientific-opinion-ema
4. Casares S, Brumeanu TD, Richie TL. The RTS,S malaria vaccine. Vaccine. 2010;28:4880–4894.
5. Miller LH, Ackerman HC, Su X, et al. Malaria biology and disease pathogenesis: insights for new treatments. Nat Med. 2013;19:156–167.
6. Rosenthal PJ. Antimalarial drug discovery: old and new approaches. J Exp Biol. 2003;206:3735–3744.
7. Enserink M. Malaria's drug miracle in danger. Science. 2010;328:844–846.
8. Wang S, Sim TB, Kim YS, et al. Tools for target identification and validation. Curr Opin Chem Biol. 2004;8:371–377.
9. Hay SI, Snow RW. The Malaria Atlas Project: developing global maps of malaria risk. PLoS Med. 2006;3:2204–2208.
10. Meissner KA, Lunev S, Wang YZ, et al. Drug target validation methods in malaria - protein interference assay (PIA) as a tool for highly specific drug target validation. Curr Drug Targets. 2016;18:470–486.

• **Comprehensive literature review describing the initial efforts to exploit oligomerisation surfaces in drug target validation.**

11. Berman HM. The Protein Data Bank. Nucleic Acids Res. 2000;28:235–242.
12. Hashimoto K, Nishi H, Bryant S, et al. Caught in self-interaction: evolutionary and functional mechanisms of protein homooligomerization. Phys Biol. 2011;8:035007.
13. Smith C. Drug target validation: hitting the target. Nature. 2003;422:341–347.
14. Jones LH. An industry perspective on drug target validation. Expert Opin Drug Discov. 2016;11:623–625.
15. Schenone M, Dančík V, Wagner BK, et al. Target identification and mechanism of action in chemical biology and drug discovery. Nat Chem Biol. 2013;9:232–240.
16. Chen XP, Du GH. Target validation: A door to drug discovery. Drug Discov Ther. 2007;1:23–29.
17. Target validation. 2019 | Sygnature Discovery. [cited 2019 Oct 28]. Available from: https://www.sygnaturediscovery.com/drug-discovery/integrated-drug-discovery/target-validation
18. Lunev S, Batista FA, Bosch SS, et al. Identification and validation of novel drug targets for the treatment of Plasmodium falciparum malaria: new insights. Curr Top Malar. 2016; 3:235–265.
    - **Literature review describing interesting target candidates that would benefit from validation by the techniques discussed in this manuscript.**
19. Edwards AM, Isserlin R, Bader GD, et al. Too many roads not taken. Nature. 2011;470:163–165.
20. Gamo F-J, Sanz LM, Vidal J, et al. Thousands of chemical starting points for antimalarial lead identification. Nature. 2010;465:305–310.
21. Guiguemde WA, Shelat AA, Bouck D, et al. Chemical genetics of Plasmodium falciparum. Nature. 2010;465:311–315.
22. Spangenberg T, Burrows JN, Kowalczyk P, et al. The open access malaria box: a drug discovery catalyst for neglected diseases. Plos One. 2013;8:e62906.
23. Santos G, Torres NV. New targets for drug discovery against malaria. PLoS ONE. 2013;8:e59968.
24. Nigussie D, Beyene T. New targets in malaria parasite chemotherapy: a review. Malaria Contr Elimination. 2015;s1:1–6.
25. Subramanian G, Belekar MA, Shukla A, et al. Targeted phenotypic screening in plasmodium falciparum and toxoplasma gondii reveals novel modes of action of medicines for malaria venture malaria box molecules. mSphere. 2018;3:1–21.
26. Cowell AN, Winzeler EA. The genomic architecture of antimalarial drug resistance. Brief Funct Genomics. 2019. DOI:10.1093/bfgp/elz008.
    - **Comprehensive review about the evolution of antimalarial resistance. It highlights the importance of genomic techniques applied to the deconvolution of inhibition mechanisms of antimalarial drugs**
27. Istvan ES, Das S, Bhatnagar S, et al. Plasmodium Niemann-Pick type C1-related protein is a druggable target required for parasite membrane homeostasis. Elife. 2019;8:1–23.
28. Fidock DA, Nomura T, Talley AK, et al. Mutations in the P. falciparum digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol Cell. 2000;6:861–871.
29. Martin RE, Marchetti RV, Cowan AI, et al. Chloroquine transport via the malaria parasite's chloroquine resistance transporter. science. 2009;325:1680–1682.
30. Veiga MI, Dhingra SK, Henrich PP, et al. Globally prevalent PfMDR1 mutations modulate Plasmodium falciparum susceptibility to artemisinin-based combination therapies. Nat Commun. 2016;7:11553.
31. Eastman RT, Dharia NV, Winzeler EA, et al. Piperaquine resistance is associated with a copy number variation on chromosome 5 in drug-pressured Plasmodium falciparum parasites. Antimicrob Agents Chemother. 2011;55:3908–3916.
32. Amaratunga C, Lim P, Suon S, et al. Dihydroartemisinin–piperaquine resistance in Plasmodium falciparum malaria in Cambodia: a multisite prospective cohort study. Lancet Infect Dis. 2016;16:357–365.
33. Nair S, Miller B, Barends M, et al. Adaptive copy number evolution in malaria parasites. PLoS Genet. 2008;4:e1000243.
34. Plowe CV, Cortese JF, Djimde A, et al. Mutations in Plasmodium falciparum dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. J Infect Dis. 1997;176:1590–1596.
35. Wu Y, Kirkman LA, Wellems TE. Transformation of Plasmodium falciparum malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. Proc Nat Acad Sci. 1996;93:1130–1134.
36. Price RN, Uhlemann A-C, van Vugt M, et al. Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant Plasmodium falciparum malaria. Clinl Infect Dis. 2006;42:1570–1577.
37. Cowman AF, Galatis D, Thompson JK. Selection for mefloquine resistance in Plasmodium falciparum is linked to amplification of the pfmdr1 gene and cross-resistance to halofantrine and quinine. Proc Nat Acad Sci. 1994;91:1143–1147.
38. Dharia NV, Plouffe D, Bopp SE, et al. Genome scanning of Amazonian Plasmodium falciparum shows subtelomeric instability and clindamycin-resistant parasites. Genome Res. 2010;20:1534–1544.
39. Korsinczky M, Chen N, Kotecka B, et al. Mutations in Plasmodium falciparumCytochrome b that are associated with atovaquone resistance are located at a putative drug-binding site. Antimicrob Agents Chemother. 2000;44:2100–2108.
40. Sutherland CJ, Laundy M, Price N, et al. Mutations in the Plasmodium falciparum cytochrome b gene are associated with delayed parasite recrudescence in malaria patients treated with atovaquone-proguanil. Malar J. 2008;7:240.
41. Ariey F, Witkowski B, Amaratunga C, et al. A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. Nature. 2014;505:50.
42. Ghorbal M, Gorman M, Macpherson CR, et al. Genome editing in the human malaria parasite Plasmodium falciparum using the CRISPR-Cas9 system. Nat Biotechnol. 2014;32:819.
43. Rungsihirunrat K, Sibley CH, Mungthin M, et al. Geographical distribution of amino acid mutations in Plasmodium vivax DHFR and DHPS from malaria endemic areas of Thailand. Am J Trop Med Hyg. 2008;78:462–467.
44. Imwong M, Pukrittakayamee S, Looareesuwan S, et al. Association of genetic mutations inPlasmodium vivax dhfr with resistance to sulfadoxine-pyrimethamine: geographical and clinical correlates. Antimicrob Agents Chemother. 2001;45:3122–3127.
45. Khim N, Andrianaranjaka V, Popovici J, et al. Effects of mefloquine use on Plasmodium vivax multidrug resistance. Emerg Infect Dis. 2014;20:1637.
46. Beghain J, Langlois AC, Legrand E, et al. Plasmodium copy number variation scan: gene copy numbers evaluation in haploid genomes. Malar J. 2016;15:206.
47. Marton MJ, Derisi JL, Bennett HA, et al. Drug target validation and identification of secondary drug target effects using DNA microarrays. Nat Med. 1998;4:1293–1301.
48. Van Spaendonk RML, Ramesar J, Van Wigcheren A, et al. Functional equivalence of structurally distinct ribosomes in the malaria parasite, Plasmodium berghei. J Biol Chem. 2001;276:22638–22647.
49. Baldi DL. RAP1 controls rhoptry targeting of RAP2 in the malaria parasite Plasmodium falciparum. Embo J. 2002;19:2435–2443.
50. Waterkeyn JG. Targeted mutagenesis of Plasmodium falciparum erythrocyte membrane protein 3 (PfEMP3) disrupts cytoadherence of malaria-infected red blood cells. Embo J. 2002;19:2813–2823.
51. Yuda M, Sakaida H, Chinzei Y. Targeted disruption of the plasmodium berghei CTRP gene reveals its essential role in malaria infection of the vector mosquito. J Exp Med. 1999;190:1711–1716.
52. Tomas AM, Margos G, Dimopoulos G, et al. The conserved P25/28 proteins of the malaria parasite surface have multiple and partially redundant functions. Embo J. 2001;20:3975–3983.
53. Ménard R, Janse C. Gene targeting in malaria parasites. Methods. 1997;13:148–157.
54. Braks JAM, Waters AP, Eling W, et al. A central role for P48/45 in malaria parasite male gamete fertility. Cell. 2004;104:153–164.
55. Nkrumah LJ, Muhle RA, Moura PA, et al. Efficient site-specific integration in Plasmodium falciparum chromosomes mediated by mycobacteriophage Bxb1 integrase. Nat Methods. 2006;3:615–621.

56. Wezena CA, Alisch R, Golzmann A, et al. The cytosolic glyoxalases of Plasmodium falciparum are dispensable during asexual blood-stage development. Microb Cell. 2018;5:32–41..
57. Crabb BS, Triglia T, Waterkeyn JG, et al. Stable transgene expression in Plasmodium falciparum. Mol Biochem Parasitol. 1997;90:131–144.
58. Carvalho TG, Ménard R. Manipulating the Plasmodium genome. Curr Issues Mol Biol. 2005;7:39–55.
59. Duraisingh MT, Triglia T, Cowman AF. Negative selection of Plasmodium falciparum reveals targeted gene deletion by double crossover recombination. Int J Parasitol. 2002;32:81–89.
60. Moolten FL. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. Cancer Res. 1986;46:5276–5281.
61. Mullen CA, Kilstrup M, Blaese RM. Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a negative selection system. Proc Natl Acad Sci U S A. 1992;89:33–37.
62. Freeman SM, Abboud CN, Whartenby KA, et al. The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. Cancer Res. 1993;53:5274–5283.
63. Moore JD. The impact of CRISPR-Cas9 on target identification and validation. Drug Discov Today. 2015;20:450–457.
64. Doyon Y, Vo TD, Mendel MC, et al. Enhancing zinc-finger-nuclease activity with improved obligate heterodimeric architectures. Nat Methods. 2011;8:74–79.
65. David Ospina-Villa J, López-Camarillo C, Castañón-Sánchez CA, et al. Advances on aptamers against protozoan parasites.
66. Dhingra SK, Redhi D, Combrinck JM, et al. A variant pfcrt isoform can contribute to plasmodium falciparum resistance to the first-line partner drug piperaquine. MBio. 2017;8:1–19.
67. McNamara CW, Lee MCS, Lim CS, et al. Targeting Plasmodium PI(4)K to eliminate malaria. Nature. 2013;504:248–253.
68. Sander JD, Dahlborg EJ, Goodwin MJ, et al. Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). Nat Methods. 2011;8:67–69.
69. Maeder ML, Thibodeau-Beganny S, Sander JD, et al. Oligomerized pool engineering (OPEN): an 'open-source' protocol for making customized zinc-finger arrays. Nat Protoc. 2009;4:1471–1501.
70. Lee MCS, Lindner SE, Lopez-Rubio -J-J, et al. Cutting back malaria: CRISPR/Cas9 genome editing of Plasmodium. Brief Funct Genomics. 2019;18:281–289.
    - **Comprehensive review aout the use of Crispr-Cas9 for *Plasmodium* genome editing.**
71. Kuang D, Qiao J, Li Z, et al. Tagging to endogenous genes of Plasmodium falciparum using CRISPR/Cas9. Parasit Vectors. 2017;10:595.
72. Wagner JC, Platt RJ, Goldfless SJ, et al. Efficient CRISPR-Cas9–mediated genome editing in Plasmodium falciparum. Nat Methods. 2014;11:915–918.
73. Zhang C, Xiao B, Jiang Y, et al. Efficient editing of malaria parasite genome using the CRISPR/Cas9 system. mBio. 2014;5:e01414.
74. Ng CL, Siciliano G, Lee MCS, et al. CRISPR-Cas9-modified pfmdr1 protects Plasmodium falciparum asexual blood stages and gametocytes against a class of piperazine-containing compounds but potentiates artemisinin-based combination therapy partner drugs. Mol Microbiol. 2016;101:381–393.
75. White J, Dhingra SK, Deng X, et al. Identification and mechanistic understanding of dihydroorotate dehydrogenase point mutations in Plasmodium falciparum that confer in vitro resistance to the clinical candidate DSM265. ACS Infect Dis. 2018;5:90–101.
76. Cradick TJ, Fine EJ, Antico CJ, et al. CRISPR/Cas9 systems targeting β-globin and CCR5 genes have substantial off-target activity. Nucleic Acids Res. 2013;41:9584–9592.
77. Kirkman LA, Lawrence EA, Deitsch KW. Malaria parasites utilize both homologous recombination and alternative end joining pathways to maintain genome integrity. Nucleic Acids Res. 2014;42:370–379.
78. Payungwoung T, Shinzawa N, Hino A, et al. CRISPR/Cas9 system in Plasmodium falciparum using the centromere plasmid. Parasitol Int. 2018;67:605–608.
79. Grindley NDF, Whiteson KL, Rice PA. Mechanisms of site-specific recombination. Annu Rev Biochem. 2006;75:567–605.
80. Russo I, Oksman A, Vaupel B, et al. A calpain unique to alveolates is essential in Plasmodium falciparum and its knockdown reveals an involvement in pre-S-phase development. Proc Nat Acad Sci. 2009;106:1554–1559.
81. Farrell A, Thirugnanam S, Lorestani A, et al. A DOC2 protein identified by mutational profiling Is essential for apicomplexan parasite exocytosis. Science. 2012;335:218–221.
82. Gabriel HB, Azevedo MF, Kimura EA, et al. Plasmodium falciparum parasites overexpressing farnesyl diphosphate synthase/geranyl-geranyl diphosphate synthase are more resistant to risedronate. Memorias Do Instituto Oswaldo Cruz. 2018;113:e180174.
83. Istvan ES, Das S, Bhatnagar S, et al. Plasmodium Niemann-Pick type C1-related protein is a druggable target required for parasite membrane homeostasis. Elife. 2019;8:e40529.
84. Charnaud SC, Dixon MWA, Nie CQ, et al. The exported chaperone Hsp70-x supports virulence functions for Plasmodium falciparum blood stage parasites. PLoS ONE. 2017;12:e0181656.
85. Florentin A, Cobb DW, Fishburn JD, et al. PfClpC Is an essential Clp chaperone required for plastid integrity and Clp protease stability in Plasmodium falciparum. Cell Rep. 2017;21:1746–1756.
86. Combe A, Giovannini D, Carvalho TG, et al. Clonal conditional mutagenesis in malaria parasites. Cell Host Microbe. 2009;5:386–396.
87. Combe A, Carvalho T, Menard R, et al. Role of Plasmodium berghei cGMP-dependent protein kinase in late liver stage development. J Biol Chem. 2009;285:3282–3288.
88. Banaszynski LA, Chen LC, Maynard-Smith LA, et al. A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. Cell. 2006;126:995–1004.
89. Armstrong CM, Goldberg DE. An FKBP destabilization domain modulates protein levels in Plasmodium falciparum. Nat Methods. 2007;4:1007–1009.
90. Absalon S, Robbins JA, Dvorin JD. An essential malaria protein defines the architecture of blood-stage and transmission-stage parasites. Nat Commun. 2016;7:11449.
91. De Koning-Ward TF, Gilson PR, Crabb BS. Advances in molecular genetic systems in malaria. Nature Rev Microbiol. 2015;13:373–387.
    - **Comprehensive overview of the molecular genetic systems available for use in Plasmodium falciparum and Plasmodium berghei.**
92. Meissner M, Krejany E, Gilson PR, et al. Tetracycline analogue-regulated transgene expression in Plasmodium falciparum blood stages using Toxoplasma gondii transactivators. Proc Nat Acad Sci. 2005;102:2980–2985.
93. Ganesan SM, Falla A, Goldfless SJ, et al. Synthetic RNA–protein modules integrated with native translation mechanisms to control gene expression in malaria parasites. Nat Commun. 2016;7:10727.
94. Goldfless SJ, Wagner JC, Niles JC. Versatile control of Plasmodium falciparum gene expression with an inducible protein-RNA interaction. Nat Commun. 2014;5:5329.
95. Polino AJ, Nasamu Armiyaw S, Niles JC, et al. Assessment of biological role and insight into druggability of the Plasmodium falciparum protease plasmepsin V. bioRxiv. 2018. DOI:10.1101/426486.
96. Garten M, Beck JR, Glushakova S, et al. Exp2 in the role of the small molecule pore in the parasitophorous vacuole membrane of Plasmodium falciparum. Biophys J. 2018;114:492a.
97. Prommana P, Uthaipibull C, Wongsombat C, et al. Inducible knockdown of plasmodium gene expression using the glmS ribozyme. PLoS ONE. 2013;8:e73783.
98. Naik RS, Krishnegowda G, Gowda DC. Glucosamine inhibits inositol acylation of the glycosylphosphatidylinositol anchors in intraerythrocytic *Plasmodium falciparum*. Journal of Biological Chemistry. 2003;278:2036–2042.
99. Elsworth B, Matthews K, Nie CQ, et al. PTEX is an essential nexus for protein export in malaria parasites. Nature. 2014;511:587–591.
100. Patel A, Perrin AJ, Flynn HR, et al. Cyclic AMP signalling controls key components of malaria parasite host cell invasion machinery. PLoS Biol. 2019;17:e3000264.

101. McLeod M, Craft S, Broach JR. Identification of the crossover site during FLP-mediated recombination in the Saccharomyces cerevisiae plasmid 2 microns circle. Mol Cell Biol. 1986;6:3357–3367.
102. Hoess RH, Ziese M, Sternberg N. P1 site-specific recombination: nucleotide sequence of the recombining sites. Proc Natl Acad Sci U S A. 1982;79:3398–3402.
103. van Schaijk BCL, Vos MW, Janse CJ, et al. Removal of heterologous sequences from plasmodium falciparum mutants using FLPe-recombinase. PLoS ONE. 2010;5:e15121.
104. Andenmatten N, Egarter S, Jackson AJ, et al. Conditional genome engineering in Toxoplasma gondii uncovers alternative invasion mechanisms. Nat Methods. 2013;10:125–127.
105. Thomas JA, Tan MSY, Bisson C, et al. A protease cascade regulates release of the human malaria parasite Plasmodium falciparum from host red blood cells. Nat Microbiol. 2018;3:447–455.
106. Phillips MA, Rathod PK. Plasmodium dihydroorotate dehydrogenase: a promising target for novel anti-malarial chemotherapy. Infect Disord Drug Targets. 2010;10:226–239.
107. Barfod A, Persson T, Lindh J. In vitro selection of RNA aptamers against a conserved region of the Plasmodium falciparum erythrocyte membrane protein 1. Parasitol Res. 2009;105:1557–1566.
108. Niles JC, Derisi JL, Marletta MA. Inhibiting Plasmodium falciparum growth and heme detoxification pathway using heme-binding DNA aptamers. PNAS. 2009;106:13266–13271.
109. Lakhin AV, Tarantul VZ, Gening LV. Aptamers: problems, solutions and prospects. Acta Naturae. 2013;5:34–43.
110. Blank M, Blind M. Aptamers as tools for target validation. Curr Opin Chem Biol. 2005;9:336–342.
111. Jana S, Paliwal J. Novel molecular targets for antimalarial chemotherapy. Int J Antimicrob Agents. 2007;30:4–10.
112. Phillips A. M, K. Rathod, P. Plasmodium dihydroorotate dehydrogenase: a promising target for novel anti-malarial chemotherapy. Infect Disord. 2010;10:226–239.
113. Coteron JM, Marco M, Esquivias J, et al. Structure-guided lead optimization of triazolopyrimidine-ring substituents identifies potent plasmodium falciparum dihydroorotate dehydrogenase inhibitors with clinical candidate potential. J Med Chem. 2011;54:5540–5561.
114. Gujjar R, El Mazouni F, White KL, et al. Lead optimization of aryl and aralkyl amine-based triazolopyrimidine inhibitors of plasmodium falciparum dihydroorotate dehydrogenase with antimalarial activity in mice. J Med Chem. 2011;54:3935–3949.
115. Ottilie S, Luth M, Winzeler E. Using in vitro evolution and whole genome analysis (IVIEWGA) to identify targets of antiparasitic compounds. ACS Infect Dis. 2018;4:301–314. DOI:10.21955/gatesopenres.1116420.1.
116. Stokes BH, Yoo E, Murithi JM, et al. Covalent Plasmodium falciparum-selective proteasome inhibitors exhibit a low propensity for generating resistance in vitro and synergize with multiple antimalarial agents. PLoS Pathog. 2019;15:e1007722.
117. Hoelder S, Clarke PA, Workman P. Discovery of small molecule cancer drugs: successes, challenges and opportunities. Mol Oncol. 2012;6:155–176.
118. Workman P, Collins I. Probing the probes: fitness factors for small molecule tools. Chem Biol. 2010;17:561–577.
119. Gotte G, Libonati M. Protein oligomerization. Oligomerization Chem Bio Compd. 2014;450. DOI:10.5772/57075.
120. Hashimoto K, Panchenko AR. Mechanisms of protein oligomerization, the critical role of insertions and deletions in maintaining different oligomeric states. Proc Nat Acad Sci. 2010;107:20352–20357.
121. Cowman AF, Healer J, Marapana D, et al. Malaria: biology and disease. Cell. 2016;167:610–624.
122. Batista FA, Bosch SS, Butzloff S, et al. Oligomeric protein interference validates drugg ability of aspartate interconversion in Plasmodium falciparum. Microbiologyopen. 2019 Feb;28:e779. .
•• **Key publication that describes the first application of the oligomeric PIA, the novel validation technique highlighted in this manuscript**
123. Nishi H, Hashimoto K, Madej T, et al. Evolutionary, physicochemical, and functional mechanisms of protein homooligomerization. Prog Mol Biol Transl Sci. 2013;117:3–24.
124. Lunev S, Butzloff S, Romero AR, et al. Oligomeric interfaces as a tool in drug discovery: specific interference with activity of malate dehydrogenase of Plasmodium falciparum in vitro. PLoS ONE. 2018;13:e0195011.
•• **Description of the *in vitro* evaluation of oligomeric distortion. Used as the basis for the development of the oligomeric PIA.**
125. Contegno F, Cioce M, Pelicci PG, et al. Targeting protein inactivation through an oligomerization chain reaction. Proc Natl Acad Sci U S A. 2002;99:1865–1869.
126. Wrenger C, Müller IB, Schifferdecker AJ, et al. Specific inhibition of the aspartate aminotransferase of plasmodium falciparum. J Mol Biol. 2011;405:956–971.
•• **The use of a peptide that mimics the oligomeric surface of target enzyme provided the initial evidence that oligomerisation-based tools have potential in the validation of target candidates.**
127. Psychogios N, Hau DD, Peng J, et al. The human serum metabolome. PLoS ONE. 2011;6:e16957.
128. Liu J, Istvan ES, Gluzman IY, et al. Plasmodium falciparum ensures its amino acid supply with multiple acquisition pathways and redundant proteolytic enzyme systems. Proc Natl Acad Sci U S A. 2006;103:8840–8845.